Improved growth of bifidobacteria by cocultivation with *Lactococcus lactis* subspecies *lactis*

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ABSTRACT

Cultivation of bifidobacteria in milk is a difficult and industrially valuable task. In this paper, we report the finding of a novel technique to improve the growth of bifidobacteria in dairy products and the results of mechanism studies. The growth of bifidobacteria in skim milk medium was found to be stimulated upon cocultivation with certain strains of *Lactococcus lactis* ssp. *lactis*. *Bifidobacterium* growth-stimulating (BGS) activity was observed on a wide range of bifidobacterial species. *Bifidobacterium* growth-stimulating activity was associated with the ability to grow in skim milk medium and the presence of a cell wall-anchored proteinase (PrtP) in *Lc. lactis* ssp. *lactis*. Studies on one strain, *Lc. lactis* ssp. *lactis* MCC857, showed that crude PrtP extracts and casein hydrolysates exhibited BGS activity. The casein hydrolysate BGS activity was found in the low molecular weight fraction by HPLC separation. A combination of 2 AA, Met and Leu, was found to account for a large portion of the casein hydrolysate BGS activity. In conclusion, this cocultivation system is highly efficient and industrially applicable for the production of fermented milk with high cell counts of bifidobacteria.

Key words: *Bifidobacterium*, *Lactococcus lactis*, growth, amino acid

INTRODUCTION

Bifidobacteria are acknowledged to be major components of the intestinal microflora of humans and animals and are frequently associated with health-promoting effects (Turroni et al., 2009). The beneficial effects of these bacteria on the intestinal flora, as well as other physiological effects, are well documented and have been recently reviewed (Leahy et al., 2005; Picard et al., 2005). For this reason, there has been increas-

ing interest in creating food products containing these bacteria as dietary probiotics.

To provide health benefits, fermented products should contain an adequate level of live probiotic bacteria at the use-by date. Assuming a daily fermented milk consumption of 100 g, the minimal level of probiotic bacteria suggested is 10^6 cfu/g based on the daily efficient dose (10^8 cfu) for these products (Vanderhoof and Young, 1998). However, the cultivation of bifidobacteria in cow milk is a difficult task compared with the cultivation of conventional starters because cow milk is an artificial medium for the growth of these nutritionally fastidious microorganisms. Low concentrations or inaccessible forms of essential nutrients in cow milk may be important factors that impair the ability of bifidobacteria to grow in cow milk. As an example, several AA (e.g., Arg, Ile, Leu, Trp, Tyr, Cys, and Val), which are either stimulatory or essential for growth of bifidobacteria and lactobacilli, are generally present in insufficient amounts at states as either free AA or low molecular mass peptides (Thomas and Pritchard, 1987; Poch and Bezkorovainy, 1988; Poch and Bezkorovainy, 1991). Therefore, cow milk-based media are usually supplemented with specific protein hydrolysates and yeast extracts to enhance the growth of bifidobacteria (Poch and Bezkorovainy, 1988; Gomes et al., 1998).

Bifidobacteria generally produce acetic acid and lactic acid from lactose, but are unable to generate sufficient acids for the manufacture of fermented milk products with the appropriate aroma and flavor. Therefore, they are normally incorporated as live cultures into fermented dairy products that can serve as carrier foods, such as yogurt, fermented by ordinary lactic acid bacterial starters, such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Samona and Robinson, 1994). Some lactic acid bacteria strains, e.g., *Lactococcus lactis*, *Lactobacillus helveticus*, *Lb. delbrueckii* ssp. *bulgaricus*, and, in rare case, *Str. thermophilus* are known to possess cell wall-anchored proteinases. Some strains of *Lc. lactis* are known to possess a cell wall-anchored proteinase (PrtP) that functions in hydrolyzing milk proteins and supplying nitrogen sources such as oligopeptides or free AA for bacterial growth

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in nonsupplemented skim milk (Juillard et al., 1995b). Therefore, we were motivated to determine whether such *Lc. lactis* strains would be capable of supplying nitrogen nutrients to bifidobacteria when they are cocultivated. In this report, we present results of improved growth of bifidobacteria by cocultivation with strains of *Lc. lactis* ssp. *lactis* together with data on mechanistic studies of *Bifidobacterium* growth-stimulating (BGS) activity of the strain MCC 857.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactococcus lactis ssp. *lactis* ATCC19435 and all bifidobacterial type strains (*Bifidobacterium longum* ssp. *infantis* ATCC15697, *Bifidobacterium breve* ATCC15700, *Bifidobacterium longum* ssp. *longum* ATCC15707, *Bifidobacterium adolescentis* ATCC15703, and *Bifidobacterium pseudocatenulatum* ATCC27919) were purchased from the American Type Culture Collection (Rockville, MD). Other strains of *Lc. lactis* were stock culture in the Morinaga Culture Collection (MCC; Morinaga Milk Industry Co. Ltd., Zama, Japan), which were obtained from dairy products. *Bifidobacterium longum* BB536 (=ATCC BAA-999) is a probiotic strain in the MCC stock that was obtained from feces of a healthy infant. Seed cultures of *Lc. lactis* ssp. *lactis* strains were grown at 30°C for 16 h in 10% (wt/wt) reconstituted skim milk (Morinaga Milk Industry Co. Ltd.) containing 0.2% (wt/wt) yeast extract (Becton and Dickinson Laboratories, Detroit, MI). Seed cultures of bifidobacterial strains were prepared in reinforced clostridial medium (Oxoid Ltd., Hampshire, UK) at 37°C for 16 h in glass test tubes. However, seed culture of *B. longum* ssp. *longum* BB536, a probiotic strain widely applied in fermented milk production, was generally prepared, as industrially, by culturing at 37°C for 4 h in 11% (wt/wt) reconstituted skim milk containing 0.2% (wt/wt) yeast extract in glass test tubes. Bacteria were enumerated by plating appropriate dilutions of each culture on transgalactosylated oligosaccharides propionate agar (Eiken Chemical Co. Ltd., Tokyo, Japan) and M17 (Oxoid Ltd.) for *Bifidobacterium* spp. and *Lc. lactis*, respectively.

Growth of *Lc. lactis* in Milk Medium

One percent of seed cultures from each strain of *Lc. lactis* ssp. *lactis* were added to 3 mL of heat-pretreated (95°C for 30 min) 10% skim milk medium in glass test tubes and incubated at 30°C for 16 h. After cultivation, medium pH was checked using a pH meter (Horiba Ltd., Kyoto, Japan) and bacteria were enumerated as described above.

Cocultivation of *Lc. lactis* with *Bifidobacteria*

First, 1% of the *B. longum* BB536 seed culture prepared in milk medium and 1% of seed cultures of each strain of *Lc. lactis* ssp. *lactis* were inoculated to 3 mL of heat-pretreated (95°C for 30 min) 10% skim milk medium in glass test tubes and incubated at 37°C for 16 h. The inoculated cell numbers were approximately 2×10^7 cfu/mL for both bifidobacteria and *Lc. lactis*.

Then, the effect of *Lc. lactis* on the growth of other bifidobacterial species was examined using the strain *Lc. lactis* ssp. *lactis* MCC857. To avoid inclusion of antioxidant reagent existing in reinforced clostridial medium, inocula of bifidobacteria were prepared in de Man, Rogosa, and Sharpe broth (Becton and Dickinson Laboratories) for 16 h at 37°C. Microorganisms were collected by centrifugation and resuspended in sterile saline to a concentration of approximately 5×10^8 cfu/mL. One percent of the bifidobacterial cell suspension and 0.1% of starter cultures of *Lc. lactis* ssp. *lactis* MCC857 were inoculated to 3 mL of heat-pretreated (95°C for 30 min) 10% skim milk medium in glass test tubes and incubated at 37°C for 16 h. After cultivation, medium pH was checked and bacteria of *Bifidobacterium* and *Lc. lactis* were enumerated as described above.

Assay of BGS Activity

Bifidobacterium growth-stimulating activity was assayed using the strain *B. longum* BB536. One percent of the *B. longum* BB536 seed culture was inoculated to heat-pretreated (95°C for 30 min) 10% reconstituted skim milk. After adding the test samples (e.g., crude PrtP solution, HPLC fractions, or AA added at appropriate ratio as described below), the inoculated medium was incubated under anaerobic condition (AnaeroPack-Anaero, Mitsubishi Gas Chemical Co. Ltd., Tokyo, Japan) at 37°C for 16 h. Incubation without sample supplementation was used as control. *Bifidobacterium* growth-stimulating activity was expressed as a stimulation index calculated by the ratio of cell numbers of bifidobacteria, determined as described above for each sample, against control.

prtP Gene Detection and Enzyme Activity Assay

Extraction of DNA from lactococcal strains was performed using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions (Qiagen Sciences, Gaithersburg, MD). Polymerase chain reaction detection of the *prtP* gene was performed as described previously (Broadbent et al., 2006). Briefly, the PCR mixture was performed using TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer's

instructions. Two sets of PCR primer pairs were used for the specific detection of *prtP* alleles encoding group b [primers GB_f (GCAAATACGGTGACGGCTGCGA) and GB2_r (TGAGCTTATAATAGGTCTTCTTCC)] and group h [primers GH_f (CAAATACGGTGACGGCTGCTAA) and GH2_r (TAGCATTATAATAGGTCTTCGTCA)]. Amplification of *prtP* fragments was achieved using a 2-min hot start at 94°C, followed by 25 cycles of denaturation at 94°C for 10 s, primer annealing at 58°C for 30 s, and extension at 72°C for 1 min. The reaction was finished with 5 min incubation at 72°C and then chilled to 4°C. Polymerase chain reaction products, which were expected to size PCR fragment of 2.7 kb, were resolved using 1.0% agarose gel electrophoresis.

Release of PrtP from *Lc. lactis* ssp. *lactis* MCC857 was performed as described previously (Laan and Konings, 1989; Exterkate and Alting, 1999). The bacteria were grown to pH 5.0 in a medium containing 10% reconstituted skim milk (pasteurized at 63°C for 30 min) and 70 mM β -glycerophosphate (sterilized at 121°C for 15 min). The cells were harvested by centrifugation ($5,000 \times g$, 10 min, 4°C) and washed twice with 30 mM sodium 2-(*N*-morpholine)ethanesulfonic acid (MES)-NaOH (pH 6.5; Sigma-Aldrich Co., St. Louis, MO) containing 10 mM CaCl₂. The cells were suspended in 1/20 culture volume of 30 mM MES-NaOH containing 10 mM EDTA-2Na for 10 min at 30°C to release the enzyme from the cell walls and centrifuged ($8,500 \times g$, 10 min, 4°C), and the supernatant was collected as a crude PrtP extract. The extract was dialyzed in 30 mM MES-NaOH containing 10 mM CaCl₂ at 4°C overnight. The crude PrtP extract was subjected to BGS assay as described above in 10% concentration. Proteinase activity was assayed with fluorescamine-labeled β -CN [0.1% (wt/vol) β -CN; Sigma-Aldrich Co.] as substrate in 30 mM MES-NaOH (pH 6.5), as described previously (Twining, 1984). Standard assays were performed at 30°C for 60 min. For inhibition of PrtP activity, a serine proteinase inhibitor, phenylmethanesulfonylfluoride (Sigma-Aldrich Co.), was added to the crude PrtP extract at a final concentration of 1 mM and incubated at room temperature for 20 min (Laan and Konings, 1989). The mixture was then assayed for BGS activity as described above.

Identification of BGS Components from PrtP-CN Hydrolysis

Cells of *Lc. lactis* ssp. *lactis* MCC857, prepared as described in isolation of PrtP, were suspended in a 1/20 culture volume of 30 mM MES-NaOH containing 10 mM CaCl₂. The cell suspension was mixed in a 1:9 ratio with 1% prepasteurized (at 85°C for 10 min) CN

(pH 6.5; Merck Inc., Whitehouse Station, NJ) and incubated at 30°C for 24 h. After incubation, the mixture was denatured by boiling for 10 min and adjusted to pH 4.6 by HCl. The mixture was centrifuged ($8,500 \times g$, 10 min, room temperature) to remove insoluble constituents. One liter of the supernatant was concentrated to 1/100 volume by freeze-drying and passed through an Amicon Ultra 3000 Da molecular filter (peptides or proteins; Millipore Co., Billerica, MA), and the permeate fraction was collected as whole hydrolysate (finally 10 mL). The hydrolysate was subjected to BGS assay as described above in 1% concentration.

The CN hydrolysate was separated by HPLC using a C₁₈ reversed-phase column (10 mm $\times$ 200 mm, 5 μ m, 100 Å; Shiseido Co. Ltd., Tokyo, Japan) with the following mobile phases: eluent A, 0.1% trifluoroacetic acid in Milli-Q water (Millipore Co.); eluent B, 0.1% trifluoroacetic acid in 80% acetonitrile (flow-rate: 4 mL/min; gradient: 0–25% acetonitrile in 10–50 min, 25–50% in 50–60 min, and 50–80% in 60–63 min). A total of 40 fractions were collected and vacuum-dried. Each fraction was dissolved in sterile distilled water at 1/40 volume of original hydrolysate and subjected to BGS assay as described above in 0.025% concentration.

Molecular mass was measured using TSQ Quantum Discovery Max (Thermo Fisher Scientific Inc., Waltham, MA). The concentration of AA was determined by using precolumn derivatization with dansyl chloride (Sigma-Aldrich Co.). The samples were hydrolyzed with 6.0 *N* HCl at 150°C for 2 h.

Statistical Analysis

All experiments were repeated in triplicate for determining standard deviation. Data were expressed as mean $\pm$ standard deviation. Data were considered statically significant when *P*-values were <0.05 , as determined by Student's *t*-test for comparisons of 2 groups or nonrepeated measures ANOVA followed by Bonferroni test for multiple comparisons with controls.

RESULTS

Stimulation of Bifidobacterial Growth in Cocultivation with *Lc. lactis*

Nineteen strains of *Lc. lactis* ssp. *lactis* were tested for BGS activity in cocultivation with *B. longum* BB536. *Bifidobacterium longum* BB536 growth was stimulated with 7 strains of *Lc. lactis* ssp. *lactis* (MCC852, MCC857, MCC858, MCC859, MCC865, MCC866, and MCC869), the medium pH values were lower than or near 4.5, and the bifidobacterial counts were approxi-

Table 1. Effect of *Lactococcus lactis* ssp. *lactis* cocultivation on bifidobacterial growth, *Lactococcus lactis* ssp. *lactis* fermentation of skim milk, and PCR detection of *prtP* genes in *Lactococcus lactis* ssp. *lactis*¹

Strain	Cocultivation with bifidobacteria ²				PCR detection of <i>prtP</i>
	pH value	Log cfu/mL of bifidobacteria	Log cfu/mL of lactococci	Milk coagulation of monoculture ³	
<i>Bifidobacterium longum</i> BB536 alone	5.49 ± 0.01	8.08 ± 0.02			
<i>Lactococcus lactis</i> ssp. <i>lactis</i> ATCC19435	4.83 ± 0.02	8.24 ± 0.03	8.36 ± 0.07	5.33 ± 0.02	–
MCC852	4.42 ± 0.05	8.96 ± 0.11**	8.67 ± 0.09	4.36 ± 0.02	+
MCC853	5.02 ± 0.03	6.36 ± 0.10	8.58 ± 0.09	5.47 ± 0.02	–
MCC854	5.29 ± 0.03	7.25 ± 0.12	8.23 ± 0.02	6.50 ± 0.02	–
MCC857	4.35 ± 0.00	8.94 ± 0.07**	8.80 ± 0.06	4.42 ± 0.03	+
MCC858	4.36 ± 0.01	9.11 ± 0.02**	8.53 ± 0.51	4.36 ± 0.01	+
MCC859	4.39 ± 0.02	9.03 ± 0.04**	8.19 ± 0.03	4.37 ± 0.02	+
MCC860	4.78 ± 0.02	8.30 ± 0.03	8.67 ± 0.07	5.39 ± 0.01	–
MCC861	4.87 ± 0.01	8.28 ± 0.08	8.43 ± 0.10	5.54 ± 0.02	–
MCC862	5.28 ± 0.02	7.38 ± 0.04	8.27 ± 0.01	6.04 ± 0.02	–
MCC863	5.50 ± 0.06	8.30 ± 0.04	8.46 ± 0.06	6.41 ± 0.04	–
MCC865	4.51 ± 0.00	8.85 ± 0.05**	8.33 ± 0.05	4.35 ± 0.03	+
MCC866	4.49 ± 0.03	8.80 ± 0.11**	8.36 ± 0.15	4.34 ± 0.03	+
MCC867	4.87 ± 0.01	8.36 ± 0.04	8.56 ± 0.05	5.53 ± 0.04	–
MCC868	5.25 ± 0.03	8.22 ± 0.01	8.11 ± 0.02	6.37 ± 0.04	–
MCC869	4.42 ± 0.01	9.05 ± 0.09**	8.03 ± 0.12	4.35 ± 0.04	+
MCC1086	5.03 ± 0.01	6.91 ± 0.51	8.39 ± 0.05	5.52 ± 0.04	–
MCC1142	4.83 ± 0.02	8.31 ± 0.05	8.66 ± 0.01	5.47 ± 0.03	–
MCC1160	4.89 ± 0.05	8.30 ± 0.02	8.65 ± 0.07	5.52 ± 0.06	–

¹Bold values indicate pH <4.6.²*Bifidobacterium longum* BB536 cultivated in skim milk alone or with each strain of *Lactococcus lactis* ssp. *lactis* at 37°C for 16 h.³Each strain of *Lactococcus lactis* ssp. *lactis* cultivated in skim milk at 30°C for 16 h.***P* < 0.01; significantly higher compared with BB536 alone.

mately 10⁹ cfu/mL, which was significantly higher (5.3- to 11-fold) compared with *B. longum* BB536 cultivated in monoculture (Table 1).

The BGS activity of MCC857, one of the most active in acid production and most favorable strains for industrial applications, was further assayed for type strains of other bifidobacterial species (Table 2). Type strains of *B. adolescentis* ATCC15703 and *B. pseudocatenulatum* ATCC27919 failed to grow in skim milk medium, with cell numbers decreasing from the inoculated numbers. However, growth of each type strain of bifidobacteria was stimulated in cocultivation of *Lc. lactis* ssp. *lactis* MCC857, with bifidobacterial cell numbers increasing 143.4-, 93.2-, 65.4-, 1.6-, 8.4-, and 44.1-fold from the inoculated cell numbers and 10.9-, 7.7-, 13.4-, 29.7-, 2,020.7-, and 11.1-fold compared with monoculture of type strains of *B. longum* ssp. *infantis* ATCC15697, *B. breve* ATCC15700, *B. longum* ssp. *longum* ATCC15707, *B. adolescentis* ATCC15703, *B. pseudocatenulatum* ATCC27919, and *B. longum* BB536, respectively.

Detection of *prtP* Genes from *Lc. lactis*

The BGS-active strains of *Lc. lactis* were found to be able to coagulate skim milk medium with pH of <4.5 (Table 1). The cell numbers in the skim milk culture

with these bacteria were around 10⁹ cfu/mL, which were higher compared with those of BGS-inactive strains (data not shown). In coincidence with the ability to grow in skim milk, these strains were found to possess the *prtP* gene as detected by PCR (Table 1).

Determination of BGS Activity in Crude *PrpP* Extracts from *Lc. lactis* ssp. *lactis* MCC857 and Identification of BGS-Active Components in *Lc. lactis* CN Hydrolysates

Proteinase activity was observed in crude *PrpP* extracts from *Lc. lactis* ssp. *lactis* MCC857 (Figure 1). Additionally, supplementation of crude *PrpP* extracts enhanced BGS activity. The addition of the serine proteinase inhibitor phenylmethanesulfonylfluoride and heat treatment blocked proteinase and BGS activity (Figure 1).

Supernatants of skim milk cultures of *Lc. lactis* were found to be negative for BGS activity (data not shown), but supernatants of milk CN incubated with *Lc. lactis*, without a supplemental carbohydrate source, were found to be BGS-active (Figure 2). *Bifidobacterium* growth-stimulating activity was present in the supernatants and the permeate fraction (molecular weight <3,000 Da) of ultrafiltration (Figure 2). Separation

Figure 1. *Bifidobacterium* growth-stimulating activity on *Bifidobacterium longum* BB536, and proteinase activity of released PrtP (a cell wall-anchored proteinase) from *Lactococcus lactis* ssp. *lactis* MCC857. The solid line indicates the proteinase activity measured by the fluorescein isothiocyanate-CN hydrolysis method. Bars show the stimulation index of BB536 in each PrtP sample: 0 min = supernatant of cell suspension before adding EDTA-2Na; 10 min = released crude PrtP fraction after incubation for 10 min with EDTA-2Na; + PMSF (phenylmethanesulfonylfluoride) = released crude PrtP fraction further incubated with 1 mM serine proteinase inhibitor PMSF at room temperature for 30 min; + boiling = released crude PrtP fraction further boiled for 10 min. *Bifidobacterium* growth-stimulating activity is shown as a stimulation index calculated by the ratio of bifidobacterial cell numbers for each sample against control without sample supplementation. The dotted line indicates the level of control.

tion of the CN hydrolysate permeate by HPLC revealed the presence of several peaks presumed to comprise a variety of peptides and free AA (Figure 3A). *Bifidobacterium* growth-stimulating activity assays showed that individual fractions were not active (data not shown). However, combined fractions exhibited BGS activity, which was found to be mainly in the hydrophilic fractions (fraction 1–10; Figure 3B). A series of assays for the screening of active combinations among fractions 1 to 10 resulted in the identification of an active combination of fractions 2 and 10. The mixture of fractions 2 and 10 accounted for approximately 60% of the BGS activity in the original permeates.

Table 3 shows the AA composition of fractions 2 and 10, as well as the whole permeates of CN hydrolysate. Fraction 2 contained mainly Val and Met, whereas frac-

Figure 2. *Bifidobacterium* growth-stimulating activity of *Lactococcus lactis* ssp. *lactis* MCC857 CN hydrolysates on *Bifidobacterium longum* BB536: 0 min = CN solution before hydrolysis; 24 h = hydrolyzed for 24 h; supernatants = supernatant of hydrolysates after centrifugation; flow-through = flow-through fraction of molecule filter with a cutoff value of 3,000 Da. *Bifidobacterium* growth-stimulating activity is shown as stimulation index calculated by the ratio of bifidobacterial cell numbers for each sample against control without sample supplementation. The dotted line indicates the level of control.

tion 10 contained mainly Leu and minor amounts of Gly and Pro. Based on MS analysis, the Gly and Pro in fraction 10 existed as a dipeptide, with the remainder as free AA.

To confirm the activity of the combination of fractions 2 and 10, BGS activity assays were performed by replacing the HPLC fractions with pure AA (Nacalai Tesque Inc., Kyoto, Japan; Figure 4). When fraction 10 was replaced with Leu, the BGS activity of the combinations did not change appreciably. When fraction 2 was replaced with Val, Met, or both, the majority of the activity was associated with the combination containing Met (data not shown). The mixture of pure Leu and Met had the same activity as the combination of fractions 2 and 10. No BGS activity was detected with other AA combinations or individual AA. These results indicated that Leu and Met were the main components associated with BGS activity in the combination of fractions 2 and 10.

Table 2. Bifidobacteria cells (log cfu/mL) in cocultivation with *Lactococcus lactis* ssp. *lactis* MCC857 or cultivated alone

Strain	After inoculation	After incubation	
		Cultivated alone	Cocultivated with <i>Lc. lactis</i> ssp. <i>lactis</i> MCC857
<i>Bifidobacterium infantis</i> ATCC15697	6.49 ± 0.09	7.61 ± 0.06	8.64 ± 0.08**
<i>Bifidobacterium breve</i> ATCC15700	6.56 ± 0.05	7.64 ± 0.06	8.53 ± 0.03**
<i>Bifidobacterium longum</i> ATCC15707	6.87 ± 0.09	7.52 ± 0.11	8.65 ± 0.12**
<i>Bifidobacterium adolescentis</i> ATCC15703	6.81 ± 0.16	5.55 ± 0.36	7.02 ± 0.09**
<i>Bifidobacterium pseudocatenulatum</i> ATCC27919	6.75 ± 0.04	4.36 ± 0.32	7.67 ± 0.12**
<i>Bifidobacterium longum</i> BB536	6.90 ± 0.06	7.50 ± 0.02	8.55 ± 0.03**

** $P < 0.01$; significant difference in bifidobacterial cell numbers of those cocultivated with *Lc. lactis* compared with those cultivated alone.

Figure 3. A) The HPLC profile of the *Lactococcus lactis* ssp. *lactis* MCC857 CN hydrolysate. B) *Bifidobacterium* growth-stimulating activity of each HPLC fraction on *Bifidobacterium longum* BB536. *Bifidobacterium* growth-stimulating activity is shown as stimulation index calculated by the ratio of bifidobacterial cell numbers for each sample against control without sample supplementation. The dotted line indicates the level of control.

DISCUSSION

In this study, we found that cocultivation with some *Lc. lactis* ssp. *lactis* strains stimulated the growth of bifidobacteria in skim milk medium. *Bifidobacterium* growth-stimulating activity was associated with the ability to grow in skim milk as well as the presence of a cell wall-anchored proteinase (Prtp) in strains of *Lc. lactis* ssp. *lactis*. Furthermore, BGS activity was observed in CN hydrolysates produced with the active strains, with Leu and Met identified as the main components that partly accounted for the BGS activity in the strain *Lc. lactis* ssp. *lactis* MCC857 CN hydrolysate.

Several reports have demonstrated the poor growth of bifidobacteria in skim or whole milk, which has been attributed to a lack of essential vitamins (Desjardins et al., 1990) or a lack of essential AA (Roy et al., 1990; Gomes et al., 1998). Various biological materials have been tested for their growth-promoting activity (such as yeast extracts, bovine CN digests, human and bovine

Figure 4. *Bifidobacterium* growth-stimulating (BGS) activity of HPLC fractions and AA on *Bifidobacterium longum* BB536. Pure Val (V), Met (M), or Leu (L) or mixtures of these were assayed for BGS activity at concentrations present in HPLC fractions 2 and 10 (final concentrations at 97, 75, and 107 μM for Val, Met, and Leu, respectively, or their mixtures). *Bifidobacterium* growth-stimulating activity is shown as a stimulation index calculated by the ratio of bifidobacterial cell numbers for each sample against control without sample supplementation. The broken line indicates the level of control.

milk whey, hog gastric mucin, and BSA digest) and were demonstrated to be effective for some bifidobacterial species, although the nature of these growth factors has not yet been well determined (Poch and Bezkorovainy, 1988; Kiviharju et al., 2005). Etoh et al. (2000) identified an active peptide for the growth of *Bifidobacterium bifidum* from natural rubber serum powder. Liepke et al. (2002) identified several bifidogenic peptides from pepsin-treated human milk. Gomes et al. (1998) reported that the growth and acid production of *Bifidobacterium animalis* ssp. *lactis* strain Bo, but not *Lactobacillus*

Table 3. Composition and concentration (μM) of AA in whole casein hydrolysate and HPLC fractions subjected to assay for *Bifidobacterium* growth-stimulating activity

Item	Whole casein hydrolysate	HPLC fraction	
		Fraction 2	Fraction 10
Asp	93	1	ND ¹
Glu	429	3	ND
Ser	64	1	ND
Thr	37	ND	ND
Gly	169	1	5
Arg	49	1	ND
Ala	137	1	ND
Pro	557	5	3
Val	311	97	ND
Met	86	75	ND
Ile	167	ND	ND
Leu	297	ND	107
Phe	145	ND	ND
Cys	16	ND	ND
Lys	210	1	ND
His	49	ND	ND
Tyr	45	ND	ND

¹ND = not detected.

acidophilus strain Ki, was promoted by proteinase- or neutrase-mediated hydrolysates. Cocultivation of *B. animalis* ssp. *lactis* Bo with *Lb. acidophilus* Ki led to enhanced rates of growth and acidification when compared with that of the individual cultures, suggesting some degree of symbiosis between the strains. They suggested that *Lb. acidophilus* Ki could hydrolyze milk CN using a cell wall-anchored proteinase to produce more low molecular mass nitrogen (especially peptides) to further growth and, consequently, aid in the growth of and acidification by *B. animalis* ssp. *lactis* Bo. Stimulatory interactions involving nitrogen requirements have previously been observed between several bifidobacterial strains and proteolytic lactobacilli (Klaver et al., 1993). To our knowledge, however, evaluation of promoting effects were restricted to certain bifidobacterial strains in these studies, and determination of the mechanisms or identification of active components have not been conducted.

Lactococci have numerous nutritional requirements for growth, particularly nitrogen sources (Reiter and Oram, 1962; Law, 1978). The AA requirement appears to be strain dependent, but most *Lc. lactis* strains require Ile, Leu, His, Met, and Val for growth (Chopin, 1993). Some strains of *Lc. lactis* are known to harbor a cell wall-anchored proteinase, PrtP, which hydrolyzes milk proteins and supplies nitrogen sources, such as various peptides and AA, for bacterial growth in milk media (Savijoki et al., 2006). The proteolytic system also includes several peptidases and 2 peptide transport systems in addition to the cell envelope-located proteinase. The gene *prtP* is generally located on plasmids (Siezen et al., 2005). The in vitro action of purified proteinases on CN has been studied in great detail (Juillard et al., 1995a). Juillard et al. (1995b) demonstrated that oligopeptides are the main source of nitrogen for *Lc. lactis* during growth in milk. Therefore, it is thought that oligopeptide transport is very likely to be the next step in the proteolytic pathway and is followed by internal degradation of the peptides by a variety of peptidase. To our knowledge, there is no report on the influence of the *Lc. lactis* proteolytic system on the growth of bifidobacteria during cocultivation. Our results showed that cocultivation of bifidobacteria with some PrtP-harboring strains resulted in markedly increased bifidobacterial cell numbers compared with bifidobacteria cultivated in monoculture. However, the effect did not appear to be mutually beneficial because bacterial counts of *Lc. lactis* were not prompted during cocultivation with bifidobacteria compared with those monocultivated at 37°C (data not shown). In addition, in experiments with supplements of Leu and Met at various concentrations to the cocultivating system,

only the bacterial counts of *B. longum*, but not *Lc. lactis*, were stimulated (data not shown).

It was expected that some nutritional components were provided from *Lc. lactis* that aided bifidobacterial growth during cocultivation; however, no BGS activity was found in the supernatants of PrtP-positive *Lc. lactis* cultured individually in milk (data not shown). These findings suggest that PrtP-positive *Lc. lactis* is highly efficient in using the hydrolysates from milk proteins and that the utilization of the active components is complete for bacteria grown in monoculture. It has been previously reported that the proteolytic system of *Lc. lactis* is negatively controlled by a rich nitrogen source (Guédon et al., 2001). However, it was found that proteinase extracts were active in promoting bifidobacterial growth. Unfortunately, the enzyme released from the cells is autoproteolytic (Laan and Konings, 1991) and loss of activity occurs during incubation. This phenomenon accounts for the relatively low BGS activity in the crude PrtP extract (Figure 1). Because preparation of milk protein hydrolysates with enzyme extracts was not practically efficient, our strategy was to treat milk proteins with *Lc. lactis* in a condition without sufficient carbohydrate source for the bacterial growth. Consequently, relatively strong BGS activities were observed in the CN hydrolysates, with the BGS activities found in the low molecular weight fractions (<3,000 Da).

High pressure liquid chromatography fractionation showed that the hydrolysates comprised numerous hydrophilic or hydrophobic peptides and AA, but no single fraction was positive for BGS activity. However, we found that BGS activity was maintained in mixtures of certain fractions and have succeeded in identifying a combination of 2 AA, Leu and Met, that account for a large portion of the BGS activity in the CN hydrolysate produced by *Lc. lactis* ssp. *lactis* MCC857. It was unexpected that the active components were AA rather than oligopeptides because it has been demonstrated that peptides identified in milk CN hydrolyzed by *Lc. lactis* are systematically longer than 4 residues, and no further extracellular degradation of the proteinase-released peptides is expected. Juillard et al. (1995a) reported the peptides released from β -CN by the action of P_I-type proteinase (PrtP) from *Lactococcus lactis* ssp. *cremoris* Wg2 contained more than 100 different oligopeptides and, with the exception of Phe, significant release of AA or di- and tripeptides could not be observed. Three types of lactococcal PrtP (P_I, P_{III}, and P_{I/III}) with different cleaving sites have been identified for *Lc. lactis* (Exterkate et al., 1993; Flambard et al., 1998). Therefore, BGS active components may be strain-dependent. However, the possible involvement of

oligopeptides in stimulating bifidobacterial growth could not be excluded. In the case of another PrtP-positive strain, MCC866, no individual or combination of AA could be identified with BGS activity (data not shown). High pressure liquid chromatography fractionation of MCC866 CN hydrolysates gave a much larger proportion of hydrophobic fractions (with later retention time, mainly composed of oligopeptides) compared with that of MCC857. *Bifidobacterium* growth-stimulating activity was distributed broadly among numerous fractions (data not shown). Therefore, we suggest that peptidase released from autolysed or physically lysed cells of strain MCC857 probably function in processing oligopeptides to AA during CN hydrolysis. Differences in the type of PrtP, composition of peptidase, and bacterial autolysis properties may thus contribute to the outcomes of various hydrolysates. Further studies are needed to evaluate the mechanisms of PrtP hydrolysis for each strain of *Lc. lactis*. Nevertheless, to our knowledge, this is the first report of the identification of bifidogenic components from milk protein hydrolysates produced by lactic acid bacteria, which may play a role in stimulating bifidobacterial growth under a cocultivation system in milk medium.

In conclusion, the stimulation of bifidobacterial growth upon cocultivation with *Lc. lactis* is a highly efficient and industrially applicable technique for the production of fermented milk with high bifidobacterial cell numbers. This technique is currently being applied in the production of a variety of fermented milk products containing probiotic *B. longum* BB536 launched in the Japanese market. Furthermore, a markedly improved survivability of bifidobacteria was also observed in cocultivated fermented milk during refrigerated storage compared with those prepared with other normal yogurt starters such as *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*. The results of the improved survivability together with mechanism studies will be reported in another paper.

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